# ROLE OF MULTI-SITE PHOSPHORYLATION IN REGULATION OF PIG HEART PYRUVATE DEHYDROGENASE PHOSPHATASE

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## 1. Introduction

Pig Heart PDH complex is phosphorylated (with MgATP) and inactivated by PDH kinase intrinsic to the complex; and reactivated by PDHP phosphatase. Fully phosphorylated PDHP complex ($\alpha_2P_3\beta_2$) contains three phosphorylated serine residues recoverable in two tryptic phosphopeptides of known amino acid sequence [1]. Inactivation is correlated with phosphorylation of one serine residue (site 1); phosphorylation of the other two serine residues inhibits reactivation by the phosphatase [1–3]. This function of the other two sites of phosphorylation has recently been questioned [4]. We define here the optimum conditions for demonstrating different rates of reactivation of pig heart PDHP ($\alpha P.\alpha\beta_2$) and PDHP ($\alpha_2P_3\beta_2$) by ox heart or pig heart phosphatase and app. $K_m$ values for PDHP complexes (substrate) and $Mg^{2+}$ and $Ca^{2+}$ (activators).

## 2. Experimental

### 2.1. *Materials*

Sources of biochemicals and radiochemicals are given in [1,5]. PDH complex was purified from pig heart [6]. PDHP complexes ($\alpha P.\alpha\beta_2$ and $\alpha_2P_3\beta_2$) were prepared with [$\gamma$-$^{32}$P]ATP [2]. The phosphorylated complexes (recovered by centrifugation; 90 min, 150 000 × *g*, 4°C) were redissolved in 20 mM potassium phosphate/2 mM DTT (pH 7.0) (equiv. 30–40 units of active complex/ml) (1 unit is 1 μmol NADH formed/min at 30° C). Each preparation was dialysed (15 h, 4°C) against 2 × 250 ml phosphate/DTT or 50 mM potassium MOPS/2 mM DTT (pH 7.0). TCA-soluble $^{32}$P was < 0.5% of total $^{32}$P. Preparations of PDHP complex ($\alpha P.\alpha\beta_2$) contained ~10% of active complex and incorporated 0.47 ± 0.045 nmol P/unit of complex inactivated (mean ± SEM for 4 preps.) with site occupancies corresponding to 85.3 ± 3.28% (site 1) 12.4 ± 2.8% (site 2) and 2.4 ± 0.48% (site 3) (determined by high-voltage electrophoresis of tryptic phosphopeptides [6]). Preparations of PDHP complex ($\alpha_2P_3\beta_2$) contained ~3% of active complex and incorporated 1.36 ± 0.115 nmol P/unit of complex inactivated (mean ± SEM for 4 preps.) with site occupancies of 36.8 ± 0.74% (site 1), 34.7 ± 1.03% (site 2) and 28.6 ± 1.27% (site 3). Preparations of PDHP complexes were free of PDHP phosphatase. PDHP phosphatase was extracted from frozen ox or pig hearts [7] and partially purified [8]. It was freed of PDH complex by two centrifugations (60 min, 150 000 × *g*, 4°C).

### 2.2. *Assays and calculations*

Active PDH complex was assayed spectrophotometrically by NADH formation [5]. PDHP complexes were assayed as PDH complex after complete conversion with phosphatase (in medium B, see below).

Rates of reactivation of PDHP complexes ($\alpha P.\alpha\beta_2$

*Abbreviations:* PDH complex, pyruvate dehydrogenase complex; PDHP, pyruvate dehydrogenase phosphate complexes; ($\alpha_2\beta_2$), tetrameric pyruvate decarboxylase (EC 1.2.4.1); PDH kinase, pyruvate dehydrogenase kinase; PDHP phosphatase, pyruvate dehydrogenase phosphate phosphatase; MOPS, 2-(*N*-morpholino) propanesulphonate; DTT, dithiothreitol; EGTA, ethanedioxybis-(ethylamine)-tetraacetate; TCA, trichloroacetic acid

and $\alpha_2P_3\beta_2$) were followed by assay of active PDH complex formed (i.e., difference from zero time). Incubations were at 30°C, (50–110 μl) and reaction initiated after 2 min preincubation by addition of substrate (or of phosphatase where $K_m$ for substrate was determined). Incubation media were: (A) 10 mM potassium phosphate/25 mM Tris–HCl/10 mM EGTA/9.75 mM $CaCl_2$/25 mM $MgCl_2$; (B) as (A) but 0.1 mM $CaCl_2$ and no EGTA; (C) 22.5 mM potassium MOPS/25 mM Tris–HCl/10 mM EGTA/9.75 mM $CaCl_2$/25 mM $MgCl_2$/1 mM potassium phosphate; (D) 47.5 mM potassium MOPS/0.1 mM $CaCl_2$/25 mM $MgCl_2$/1 mM potassium phosphate; (E) 44 mM potassium MOPS/0.1 mM $CaCl_2$/10 mM $MgCl_2$/2.5 mM potassium phosphate. All media contained 2 mM DTT and were at pH 7.0.

$CaCl_2$ in stock solutions and incubation media was assayed by atomic absorption spectrometry against a primary $CaNO_3$ standard (BDH Ltd., Poole, Dorset). Concentrations of free $Ca^{2+}$ and $Mg^{2+}$ were computed from dissociation constants [8].

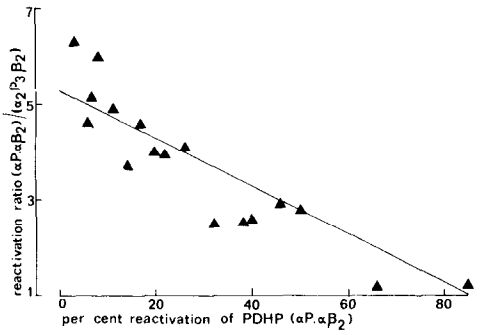


Fig.1. Incubations were made in medium A (see section 2.2); computed [$Mg^{2+}$] 17 mM, [$Ca^{2+}$] 13 μM, [($\alpha P.\alpha\beta_2$)] 2.9 units/ml, [($\alpha_2P_3\beta_2$)] 3.0 units/ml. Samples were taken for assay of active PDH complex at times ranging from 1–20 min. There was a total of 87 obs.; values showing (≤ 2%) the same percentage hydrolysis were averaged in the figure but not in the least squares linear regression analysis. Slope −0.05 ± 0.008 (mean ± SEM for ratio/% reactivation); intercept 5.28 ± 0.293 (ratio at zero reactivation); $r$ (correlation coefficient) −0.684 ($P < 0.001$).

## 3. Results and discussion

The results shown in fig.1,2 and table 1 were obtained with pig heart PDHP complexes and ox heart phosphatase (ox heart phosphatase was more readily available and was more stable on storage than pig heart phosphatase). The results described have been duplicated with pig heart phosphatase.

Table 1
Effect of incubation medium on rates of reactivation of pig heart PDHP complex ($\alpha P.\alpha\beta_2$ and $\alpha_2P_3\beta_2$) by ox heart PDHP phosphatase

| Incubation medium | Ratio of rates of formation of active PDH complex; mean ± SEM for (rate with $\alpha P.\alpha\beta_2$)/(rate with $\alpha_2P_3\beta_2$) at: | | |
|---|---|---|---|
| | 5–20% reactivation | 20–30% reactivation | 30–60% reactivation |
| A | 4.66 ± 0.25 (39) | 3.56 ± 0.21 (24) | 2.72 ± 0.20 (12) |
| B | 4.77 ± 0.73 (8) | 3.26 ± 0.54 (8) | 2.69 ± 0.17 (11) |
| C | 5.79 ± 1.23 (8) | 3.51 ± 0.28 (5) | – |
| D | 2.26 ± 0.19 (20)[a,b] | 1.58 ± 0.13 (9)[a,b] | – |
| E | 1.29 ± 0.20 (4)[a,b] | 1.25 ± 0.15 (4)[a,b] | 1.18 ± 0.03 (2) |

[a] $P < 0.001$ for (A–D) and (A–E)
[b] $P < 0.05$ for (B–D) and (B–E)

For composition of media see section 2.2. Concentrations: (A) $Mg^{2+}$ 16.6 mM, $Ca^{2+}$ 13 μM, ($\alpha P.\alpha\beta_2$) 2.85 units/ml, ($\alpha_2P_3\beta_2$) 2.90 units/ml; (B) $Mg^{2+}$ 16.7 mM, $Ca^{2+}$ 79 μM, ($\alpha P.\alpha\beta_2$) 3.37 units/ml, ($\alpha_2P_3\beta_2$) 3.54 units/ml; (C) $Mg^{2+}$ 24 mM, $Ca^{2+}$ 15.3 μM, ($\alpha P.\alpha\beta_2$) 2.25 units/ml, ($\alpha_2P_3\beta_2$) 2.1 units/ml; (D) $Mg^{2+}$ 24 mM, $Ca^{2+}$ 98 μM, ($\alpha P.\alpha\beta_2$) 2.1 units/ml, ($\alpha_2P_3\beta_2$) 2.1 units/ml; (E) $Mg^{2+}$ 8.2 mM, $Ca^{2+}$ 90 μM, ($\alpha P.\alpha\beta_2$) 2.0 units/ml, ($\alpha_2P_3\beta_2$) 2.0 units/ml. PDHP complexes added in phosphate/DTT (A,B) or MOPS/DTT (C–E). Number of observations in parentheses

### 3.1. *Rates of reactivation of PDHP complexes ($\alpha P.\alpha\beta_2$ and $\alpha_2P_3\beta_2$); effect of extent of reactivation*

As shown in fig.1, there was an inverse correlation between the ratio of reactivation $(\alpha P\alpha\beta_2)/(\alpha_2P_3\beta_2)$ and the % reactivation of $(\alpha P\alpha\beta_2)$. As analysed by least squares linear regression analysis the intercept (ratio at zero reactivation) was 5.28 ± 0.29 (mean ± SEM). Thus the initial rate of reactivation of PDHP complex $(\alpha P\alpha\beta_2)$ by phosphatase was ~5-times that of fully phosphorylated PDHP complex $(\alpha_2P_3\beta_2)$. The data was generated by incubating partial and fully phosphorylated complexes for fixed times ranging from 1–20 min under identical conditions. There was a total of 87 obs. but these have been condensed by pooling in fig.1 for ease of presentation. Results with pig heart phosphatase (not shown) were very similar (intercept, 3.92 ± 0.25; slope, −0.07 ± 0.015; $r$, −0.985; 15 obs.). The results and conclusions in [2] are thus confirmed.

### 3.2. *Effect of incubation medium on relative rates of reactivation of PDHP complexes by phosphatase*

Teague et al. [4] failed to show significant differences in rates of reactivation of partial and fully phosphorylated bovine kidney PDHP complexes with bovine kidney phosphatase. MOPS buffers and 0.1 mM-$CaCl_2$ were used in [4] whereas in [2] phosphate/Tris buffer with Ca-EGTA buffer was used. As shown in table 1 (medium E) there was no significant difference in rates of reactivation of pig heart PDHP complexes by ox heart phosphatase employing the incubation medium in [4] (i.e., the ratio of rates of reactivation was not significantly different from unity). Under all other incubation conditions investigated, the rate of reactivation of partially phosphorylated PDHP complex $(\alpha P\alpha\beta_2)$ was significantly greater than that of fully phosphorylated complex $(\alpha_2P_3\beta_2)$. Increasing $MgCl_2$ from 10–25 mM in the incubation medium of [4] produced a modest increase in the ratio of rates of reactivation (medium D in table 1). The highest ratios were seen in phosphate/Tris buffers employing either a Ca-EGTA buffer (medium A) or 0.1 mM $CaCl_2$ (medium B) or in a potassium MOPS/Tris buffer with Ca-EGTA buffer (medium C in table 1). In table 1, ratios of rates of reactivation are shown for 5–20%, 20–30% and 30–60% reactivation of partially phosphorylated PDHP complex. These were generated by incubating partial and fully phosphorylated complexes under identical conditions for fixed times ranging from 1–20 min. The differences between the results in [4] and [2] would thus appear to be due to differences in incubation media. The negative findings with medium E appear to result from a combination of MOPS buffer, 0.1 mM $CaCl_2$ in place of Ca-EGTA and of 10 mM $MgCl_2$ in place of 25 mM $MgCl_2$. Addition of KCl to 20 mM or of potassium phosphate to 9 mM in medium E did not increase the ratio (not shown). Addition of MOPS to 50 mM in medium A did not change the ratio (not shown).

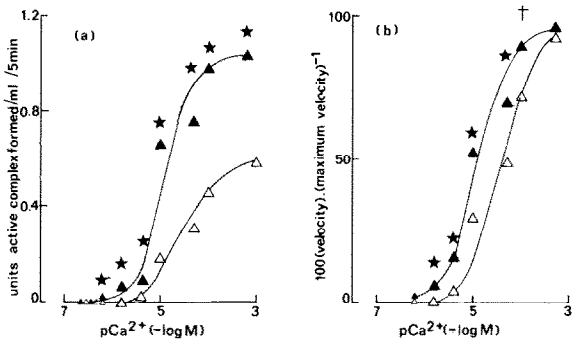


Fig.2. Incubations were made in 10 mM potassium phosphate/25 mM Tris–HCl/2 mM DTT (pH 7.0). Computed $[Mg^{2+}]$ 25 mM in all incubations; computed $[Ca^{2+}]$ as $pCa = -\log[Ca^{2+}]$ shown on abscissa. For $pCa^{2+}$, 6.57–4.97, CaEGTA buffers (20 mM EGTA); for $pCa^{2+}$, 4.30–3.30, $CaCl_2$. For calculation of $[Mg^{2+}]$ and $[Ca^{2+}]$ see section 2.2. Concentrations of PDHP complexes were 3.2 units/ml (▲,$\alpha P.\alpha\beta_2$) and 3.4 units/ml (△,$\alpha_2P_3\beta_2$). Samples for assay of active complex were taken after 5 min; progress curves were linear over this period (not shown). The $V_{max}$ values used in panel (b) were computed from the data in panel (a) and were 1.08 ± 0.71 ($\alpha P.\alpha\beta_2$) and 0.63 ± 0.053 ($\alpha_2P_3\beta_2$) (mean ± SEM units of active complex formed/ml/5 min). Each point mean of 5 obs. $^*P < 0.01$; $^\dagger P < 0.05$ for difference [(▲,$\alpha P.\alpha\beta_2$)−(△,$\alpha_2P_3\beta_2$)].

### 3.3. *Effect of concentrations of PDHP complexes, $Mg^{2+}$ and $Ca^{2+}$ on rates of reactivation*

In these experiments active PDH complex was assayed after 5 min incubation (control experiments showed linear progress curves for 5 min at highest rates of reactivation). The app. $K_m$ for both PDHP complexes was 22.2 units/ml (as equivalent PDH complex); $V_{max}$ values were 3.03 ($\alpha P.\alpha\beta_2$) and 0.81 ($\alpha_2P_3\beta_2$) (in units PDH complex formed/ml incubate/

5 min). The ratio of $V_{max}$ values was thus 3.7 (medium A; 4 concentrations of PDHP complexes, range 2.3 25.2 units/ml; 5 obs. each concentration). The app. $K_m$ for $Mg^{2+}$ was 0.59 mM (both PDHP complexes) and $V_{max}$ values were 1.56 ($\alpha P.\alpha\beta_2$) and 0.84 ($\alpha_2P_3\beta_2$). The ratio of $V_{max}$ values was 1.86 [medium B; four $Mg^{2+}$ values over 0.64–10 mM; PDHP ($\alpha P.\alpha\beta_2$) 2.76 units/ml, PDHP ($\alpha_2P_3\beta_2$) 3.56 units/ml; 10 obs. at each $Mg^{2+}$ concentration].

The effect of $Ca^{2+}$ concentration on rates of reactivation is shown in fig.2(a). Neither preparation of PDHP complex showed significant reactivation in the absence of added $Ca^{2+}$. The concentrations of $Ca^{2+}$ required for significant reactivation were 0.64 $\mu$M ($\alpha P.\alpha\beta_2$) and 3.8 $\mu$M ($\alpha_2P_3\beta_2$). The $V_{max}$ values (computed by the method in [9]) were 1.08 ± 0.071 ($\alpha P.\alpha\beta_2$) and 0.63 ± 0.053 ($\alpha_2P_3\beta_2$) (mean ± SEM for units PDH complex formed/ml/5 min; $P$ for difference $< 0.001$). The ratio of $V_{max}$ values was 1.71 ± 0.14 ($P < 0.001$ for difference from unity). Computed app. $K_m$ values were 15.6 ± 3.45 $\mu$M ($\alpha P.\alpha\beta_2$) and 43.8 ± 11.42 ($\alpha_2P_3\beta_2$). The difference is not statistically different. As shown in fig.2(b) the observed values of (rate of reactivation) as a fraction of $V_{max}$ at each $Ca^{2+}$ concentration below 500 $\mu$M were significantly greater for ($\alpha P.\alpha\beta_2$) than for ($\alpha_2P_3\beta_2$). This would appear to show that the app. $K_m$ for $Ca^{2+}$ for reactivation of ($\alpha P\alpha\beta_2$) by ox heart phosphatase is lower than for reactivation of ($\alpha_2P_3\beta_2$). These experiments have been repeated with pig heart phosphatase with closely similar results ($V_{max}$ values 0.42 ± 0.059 and 0.24 ± 0.05; app. $K_m$ values 26.6 ± 12.6 $\mu$M and 116.1 ± 64.3 $\mu$M $Ca^{2+}$).

The app. $K_m$ values for $Ca^{2+}$ in the present study are much higher than the value of ~0.7 $\mu$M $Ca^{2+}$ obtained in [10] but the app. $K_m$ for $Mg^{2+}$ was similar. This increase in $K_m$ for $Ca^{2+}$ is borne out by the higher app. $K_m$ for PDHP complex in the present study (22 units/ml at 13 $\mu$M $Ca^{2+}$) (2.5 units/ml at 10 $\mu$M $Ca^{2+}$ in [10]). $Ca^{2+}$ lowers the app. $K_m$ for the PDHP complex [10,11]; the rise in app. $K_m$ for $Ca^{2+}$ should therefore increase the app. $K_m$ for PDHP complex at the $Ca^{2+}$ concentration employed. The higher app. $K_m$ for $Ca^{2+}$ in the present study is attributed to the new method of PDH complex purification [6] used. Depletion of PDHP complexes of $Ca^{2+}$ increases the app. $K_m$ for $Ca^{2+}$ [10] and the new method of preparation of PDH complex (and hence of PDHP complex) [6] should lead to greater depletion of $Ca^{2+}$ than the method used in [5,10]. The studies [2] which first showed that phosphorylation of sites 2 and 3 in the pig heart PDHP complex inhibits reactivation by phosphatase used PDH complex prepared by the old method [5] and showed the expected higher sensitivity to $Ca^{2+}$ in the phosphatase reaction.

This study has shown that the inhibitory effect of site 2 and site 3 phosphorylations on reactivation of PDHP complex by phosphatase is demonstrable at $V_{max}$ for PDHP complexes (substrates) and for $Mg^{2+}$ or $Ca^{2+}$ (activators). It has been shown that phosphorylation of sites 2 and 3 specifically increases app. $K_m$ for $Ca^{2+}$ thus emphasising the potential significance of mitochondrial $Ca^{2+}$ concentration as a regulator of PDH complex activity in vivo. It would seem important to establish the mitochondrial concentration of $Ca^{2+}$ and the $K_m$ for $Ca^{2+}$ of the phosphatase reaction in mitochondria.

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**References**

[1] Sugden, P. H., Kerbey, A. L., Randle, P. J., Waller, C. A. and Reed, K. B. M. (1979) Biochem. J. 181, 419–426.
[2] Sugden, P. H., Hutson, N. J., Kerbey, A. L. and Randle, P. J. (1978) Biochem. J. 169, 433–435.
[3] Sugden, P. H. and Randle, P. J. (1978) Biochem. J. 173, 659–668.
[4] Teague, W. M., Pettit, F. H., Yeaman, S. J. and Reed, L. J. (1979) Biochem. Biophys. Res. Commun. 87, 244–252.
[5] Cooper, R. H., Randle, P. J. and Denton, R. M. (1974) Biochem. J. 143, 625–641.
[6] Kerbey, A. L., Radcliffe, P. M., Randle, P. J. and Sugden, P. H. (1979) Biochem. J. 181, 427–433.
[7] Siess, E. A. and Wieland, O. H. (1972) Eur. J. Biochem. 26, 96–105.
[8] Severson, D. L., Denton, R. M., Pask, H. T. and Randle, P. J. (1974) Biochem. J. 140, 225–237.
[9] Jones, A. (1970) Comput. J. 13, 301–308.
[10] Randle, P. J., Denton, R. M., Pask, H. T. and Severson, D. L. (1974) Biochem. Soc. Symp. 39, 75–87.
[11] Pettit, F. H., Roche, T. E. and Reed, L. J. (1972) Biochem. Biophys. Res. Commun. 49, 563–571.